

Pregnant Female Lizards *Iberolacerta cyreni* Adjust Refuge Use to Decrease Thermal Costs for Their Body Condition and Cell-Mediated Immune Response

LUISA AMO*, PILAR LÓPEZ, AND JOSÉ MARTÍN

Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales, CSIC, 28006 Madrid, Spain

ABSTRACT Lizards often respond to increased predation risk by increasing refuge use, but this strategy may entail a loss of thermoregulatory opportunities, which may lead to a loss of body condition. This may be especially important for pregnant oviparous female lizards, because they need to maintain optimal body temperatures as long as possible to maximize developmental embryos rate until laying. However, little is known about how increased time spent at low temperatures in refuges affects body condition and health state of pregnant female lizards. Furthermore, it is not clear how initial body condition affects refuge use. Female Iberian rock lizards forced to increase time spent at low temperatures showed lower body condition and tended to show lower cell-mediated immune responses than control females. Therefore, the loss of thermoregulatory opportunities seems to be an important cost for pregnant females. Nevertheless, thereafter, when we simulated two repeated predatory attacks, females modified refuge use in relation to their body condition, with females with worse condition decreasing time hidden after attacks. In conclusion, female lizards seemed able to compensate increased predation risk with flexible antipredatory strategies, thus minimizing costs for body condition and health state. *J. Exp. Zool.* 307A:106–112, 2007.

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Many prey respond to predators by increasing refuge use (Sih et al., '92; Martín and López, '99a,b). However, available or safer refuges, such as rock crevices, may be in microhabitats with shadier and cold conditions. Thus, the body temperature of an ectothermic animal may decrease below optimal levels after being hidden inside a cold refuge (Polo et al., 2005). This is especially important for small animals, such as lizards, with a low thermal inertia because it could result in temperature impairment in only a few minutes. In reptiles, the maintenance of an optimal body temperature is essential to maximize physiological processes (Huey, '82; Stevenson et al., '85). This may be related to the finding that intensive refuge use leads to a loss of body condition in lizards (Martín and López, '99a). Low temperatures inside refuges may be especially costly for pregnant females as they need to maintain optimal body temperatures to maximize

developmental rates of embryos (Mathies and Andrews, '97; Shine, 2004), which are temperature dependent (Muth, '80). Thus, increased refuge use by pregnant females can not only decrease their body condition, but can also negatively affect their offspring.

Nutritional status can also influence the capacity of lizards to mount an immune response to infection (Cooper et al., '85; Smallridge and Bull, 2000). Decreased body condition due to increased refuge use may influence host–parasites relationships, and may increase the negative effects of

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*Correspondence to: L. Amo, Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales, CSIC, José Gutiérrez Abascal 2, 28006 Madrid, Spain. E-mail: luisa.amo@mncn.csic.es

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parasites on their host. Therefore, although refuges may prevent prey to be captured, thermal costs of refuge use may have consequences for long-term fitness of prey.

Theoretical models of refuge use suggest that prey should adjust time spent in a refuge so that the optimal emergence time is the time when the costs of staying exceed the costs of leaving (Sih et al., '92; Martín and López, '99b; Polo et al., 2005). The decision of when to come out from a refuge should be optimized by considering the expected fitness effects of diminution of risk with time in the exterior, but also considering costs of refuge use. Hence, animals should accurately adjust refuge use to cope with increased predation risk without incurring excessive costs (Sih, '92, '97; Dill and Fraser, '97; Martín and López, '99b; Martín et al., 2003a,b). Previous studies have examined the ability of prey to modify refuge use to cope with risk without incurring costs in terms of loss of time to perform other activities (Sih, '97; Martín et al., 2003a,b). However, little is known about whether prey use refuges according to their initial body condition to minimize physiological costs that may affect their body condition.

The Iberian rock lizard, *Iberolacerta cyreni* (= *Lacerta monticola cyreni*) is a small diurnal lacertid found mainly in rocky habitats in high mountains of the Iberian Peninsula, where temperatures are limiting for lizards. These lizards responded to predators by rapidly running for cover into the nearest refuge, usually rock crevices (Carrascal et al., '92). Due to low environmental temperatures, thermal costs of refuges may be very high for this species (Martín and López, '99b; Polo et al., 2005). This cost should be especially important for pregnant females, that have to maintain optimal body temperatures during the 1-month gestation of embryos until egg laying.

Here we performed a laboratory study to analyze the effects of time spent at low temperatures, due to intensive refuge use, on body condition, health state and parasite load of pregnant female lizards, *I. cyreni*. We predicted that females forced to increase time spent at low temperatures, simulating temperatures inside cold refuges, would decrease their body condition and their cell-mediated immune (CMI) response and increase parasite loads. We further analyzed whether pregnant female lizards modify refuge use in relation to their current body condition. We hypothesized that, under the same levels of risk, lizards with a compromised body condition would spend less time hidden in refuges after a pre-

dator's attack, whereas lizards in good body condition would spend more time hidden in refuges. Alternatively, if avoiding predation was more important than these associated physiological costs, initial body condition should not affect refuge use.

MATERIAL AND METHODS

Study area and species

In July 2004, we captured by noosing 20 adult female *I. cyreni* lizards in the Guadarrama Mountains (Madrid Prov., Central Spain) at an elevation range of 1,900–2,200 m. Natural landscape is characterized by granite rock boulders and scree interspersed with shrubs (*Cytisus oromediterraneus* and *Juniperus communis*), together with meadows of *Festuca* and other grasses (Martín and Salvador, '97). In this region *I. cyreni* (snout-to-vent length, SVL, of adult lizards range between 65 and 90 mm) is active from May to September due to limiting environmental temperatures. Lizards mate in May–June, and oviparous females produce a single clutch, in July–August, that ranges between 5 and 8 eggs (Palacios and Salvador, '74; Elvira and Vigal, '85; Pérez-Mellado, '98). Most body fat stores of females are invested in vitellogenesis and formation of the clutch (Braña et al., '92), with clutch size and weight increasing when female body size is larger. When captured, females had mating scars on the belly indicating several copulations. At the end of the experiment, we confirmed that all females were actually pregnant by abdominal palpation.

Lizards were individually housed at "El Ventorrillo" Field Station 5 km from the capture site, in outdoor 60 × 40 cm PVC terraria containing sand substratum and rocks for cover. Water was provided ad libitum. The photoperiod and ambient temperatures were those of the surrounding region. To avoid changes in body and health condition, and parasite load of lizards due to captivity per se, they were held in captivity only 1 week before testing to allow acclimation to laboratory conditions. After the experiment, lizards were released at the points of capture.

We weighed and measured SVL of lizards immediately after capture. To assess blood parasite load, we made a smear on a microscope slide from blood taken from the postorbital sinus by using one 9 µl heparinized hematocrit tube. Blood smears were air-dried, fixed in absolute methanol for 10 min and then stained in Giemsa diluted 1:9

with phosphate buffer (pH 7.2) for 40 min before their examination for parasites. On mounted slides, number of intraerythrocytic blood parasites was estimated at $1,000 \times$ by counting the number of parasites per 2,000 erythrocytes. The only parasites found were hemogregarines (Amo et al., 2004).

We measured CMI responsiveness to lizards by using a delayed-type hypersensitivity test. This test is a reliable measure of cell-dependent immunocompetence in vivo (Lochmiller et al., '93), and it has been used in many studies of animals including lizards (Merino et al., '99; Svensson et al., 2001; Belliure et al., 2004; Amo et al., 2006). CMI responsiveness was estimated on the basis of quantification of the swelling response to intradermally injected phytohemagglutinin (Smits et al., '99). We injected the lizard's footpad of the right hind limb with 0.02 ml of phytohemagglutinin solution (PHA-P, Sigma), and measured the swellings with a pressure-sensitive spessimeter (to the nearest 0.01 mm) before and 24 hr after the injection (Smits et al., '99). Results of previous studies showed that repeatability of this measure, calculated as the intraclass correlation coefficient (Lessells and Boag, '87) was high ($r > 0.95$, L. Amo, unpublished data). These measures were taken again immediately after the experiments finished. Although, response to PHA injection is not well known in lizards, and the immune system of lizards might respond differently to the first than to the second injection of PHA. In any case, the possible variations should be similar for individuals of all treatments, and, thus, any differences between groups should be attributed to the effect of treatment.

Females of similar body size were randomly assigned to two treatments. Initial body size of females did not differ between treatments (SVL: mean \pm SE: control: 74 ± 1 mm, experimental: 74 ± 1 mm, ANOVA, $F_{1,18} = 0.01$, $P = 0.94$; Body mass: control: 6.7 ± 0.3 g, experimental: 6.6 ± 0.3 g, ANOVA, $F_{1,18} = 0.06$, $P = 0.80$). In the "control" treatment, lizards were maintained all day in outdoor conditions (mean \pm SE; T_a : $21.6 \pm 0.4^\circ\text{C}$, T_s : $28.7 \pm 0.6^\circ\text{C}$) where they could normally bask and attain optimal body temperatures. In the "no thermoregulation" treatment, lizards were maintained outdoor as above, but their terraria were moved inside a cold room (mean \pm SE; T_a : $16.5 \pm 0.1^\circ\text{C}$, T_s : $16.4 \pm 0.1^\circ\text{C}$) during 4 hr, from 8:00 to 12:00 GTM every day. Thereafter, terraria were replaced to outdoor conditions. To avoid differences in disturbance regimes due to the

movement of experimental terraria, we also moved control terraria within the outdoor area at the same times than experimental terraria. All lizards were fed two mealworms at 12:30 GTM each day, and we ensured that all lizards ate them. Thus, all females could made food digestion under normal outdoor conditions. This procedure was repeated during 10 consecutive days.

We used repeated-measures two-way ANOVAs to analyze differences in body mass between the beginning and the end of the experimental treatments ("time"; within-subject factor) and between treatments (between-subject factor), including the interaction to test for differences between treatments in the course of time. We used repeated-measures two-way ANOVAs to examine differences in body mass, CMI response or parasite load between the beginning and the end of the experimental treatments ("time"; within-subject factor) and between treatments (between-subject factor), including the interaction to test for differences between treatments in the course of time.

Immediately after these treatments finished, we designed an experiment to test whether time spent hidden inside refuges by lizards after two successive simulated predator attacks depended on their body condition. The experiment was conducted in a terrarium ($100 \times 40 \times 50$ cm) with a sand substrate and a single refuge in the middle of one end of the terrarium. The refuge was built with flat rocks, which had one opening (7×6 cm). Air temperature inside the refuge during tests was maintained at $17.4 \pm 0.1^\circ\text{C}$ to avoid the confounding effects of temperature differences on refuge use (Martín and López, '99b).

Lizards were gently transferred to the experimental terraria, where the refuge had the door initially closed, and given 2 min before trials for acclimatization to a novel environment. Then, the experimenter opened the door of the refuge and simulated a predatory attack by tapping lizards close to the tail with a brush to stimulate them to run and hide in the refuge. When the lizard hid, we retreated to a hidden position and recorded the time that the lizard spent in the refuge until the lizard emerged entirely from the refuge ("emergence time"). Immediately after the lizard resumed normal activity, we simulated another predatory attack with the same procedure and measurements as in the first attack.

We used repeated-measures two-way ANOVAs to test for differences in emergence time from the refuge between the first and the second attack (within-subject factor) and between treatments

(between-subject factor). We included the interaction to test for differences between treatments between attacks.

RESULTS

Body mass of female lizards differed significantly between the beginning and the end of the experiment (repeated-measures ANOVA, $F_{1,18} = 198.90$, $P < 0.0001$). There were not significant overall differences between treatments ($F_{1,18} = 0.57$, $P = 0.46$), but the interaction between time and treatment was significant ($F_{1,18} = 7.53$, $P = 0.01$; Fig. 1a). Thus, all females increased their body mass in the course of the experiment, but control

females increased their body mass more than experimental ones.

Blood parasite load was significantly higher at the end of the experiment in both treatments (repeated-measures ANOVA, $F_{1,18} = 13.57$, $P = 0.002$), experimental females tended to be more parasitized than control females ($F_{1,18} = 3.85$, $P = 0.07$), but the interaction was not significant ($F_{1,18} = 0.90$, $P = 0.36$, Fig. 1b). Thus, the experimental treatments did not seem to affect blood parasite load of females.

After the experiment finished, females showed significantly lower CMI responses than before the experiment (repeated-measures ANOVA, $F_{1,18} = 6.49$, $P = 0.02$). There were no overall sig-

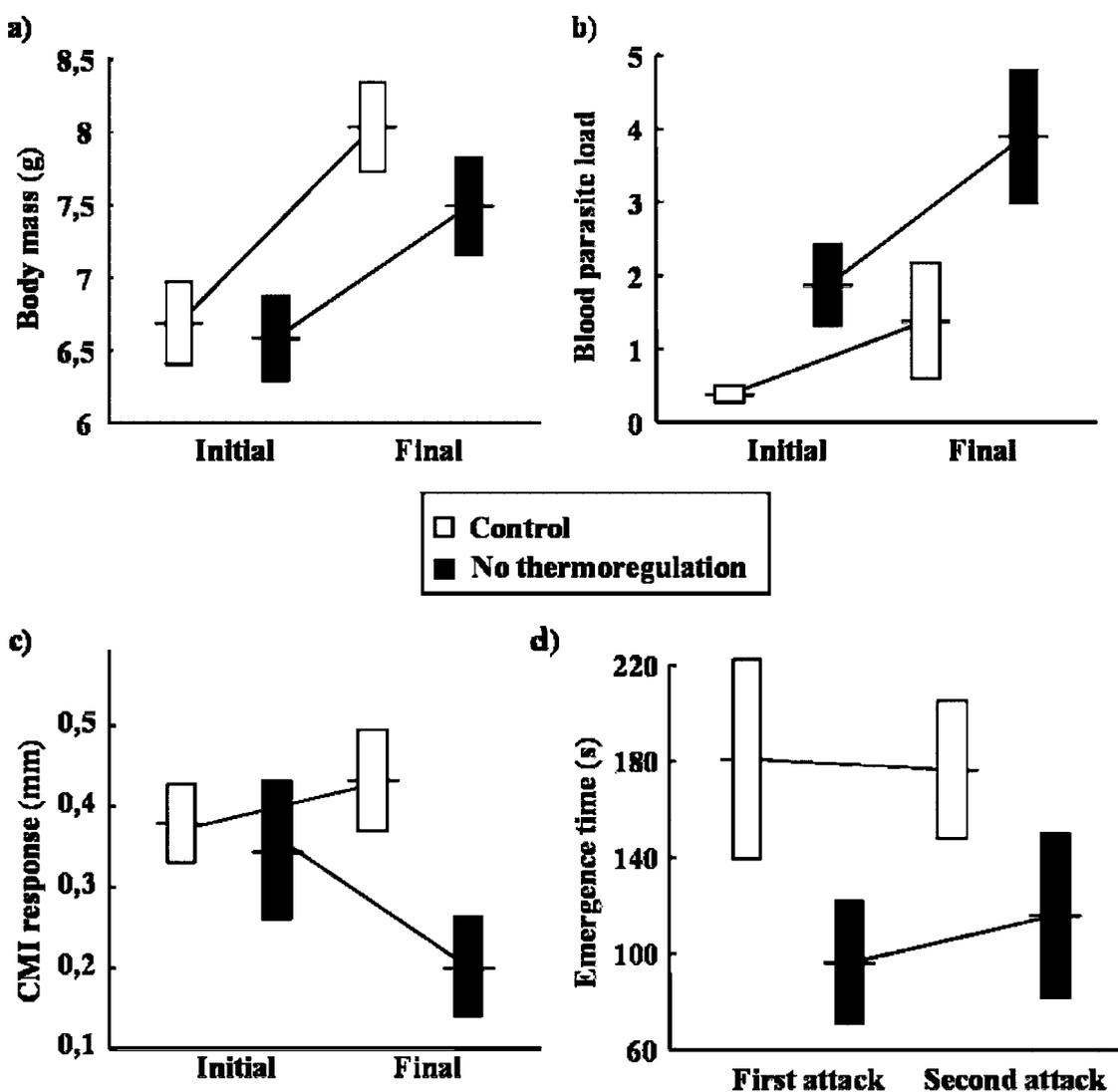


Fig. 1. Mean (\pm SE) (a) body mass (g), (b) blood parasite load (no. infected cells/2,000 erythrocytes) and (c) T-cell-mediated immune response—CMI (mm) of pregnant female lizards, *Iberolacerta cyreni* before and after an experiment testing for the effects of costs of thermoregulation inside refuges (see methods). (d) Mean (\pm SE) emergence time (s) of female lizards when they suffered two simulated repeated attacks after the experimental treatments.

nificant differences between treatments ($F_{1,18} = 0.61$, $P = 0.45$), but the interaction approached significance ($F_{1,18} = 3.18$, $P = 0.09$, Fig. 1c). Thus, control females did not significantly change their CMI response during the experiment (Tukey's test, $P = 0.95$), whereas experimental females significantly decreased their CMI ($P = 0.03$).

In relation to refuge use after the simulated attacks, experimental females tended to emerge sooner from the refuge than control females (repeated-measures ANOVA, $F_{1,17} = 4.09$, $P = 0.06$). There were no differences between attacks ($F_{1,17} = 0.56$, $P = 0.47$) and the interaction was not significant ($F_{1,17} = 0.01$, $P = 0.99$; Fig. 1d).

DISCUSSION

Our study suggests that increased refuge use results in a decrease in thermoregulatory opportunities, that, in turn, affects body condition and health of pregnant female *I. cyreni*. Experimental females that were maintained for longer periods under low temperatures (simulating conditions inside cold refuges) did not increase their body mass as much as control females, which could normally bask and attain optimal body temperatures. Moreover, experimental females tended to show a lower CMI response than control females. These results agree with previous studies with juvenile *Crocodylus porosus* crocodiles that showed depressed total white cell counts after being submitted to low temperatures (Turton et al., '97).

The ability to mount a CMI response to a mitogenic stimulus may have important fitness consequences (Gonzalez et al., '99), because it constitutes a generalized short-term response to grafts, allergens and wounds. Furthermore, as female skinks *Egernia whitii* seem to have reduced numbers of lymphocytes during pregnancy than afterwards (Cartledge et al., 2005), a greater decrease in the CMI response of experimental female lizards in our study suggests that females submitted to low temperatures may also suffer immunological costs. As the CMI response may be implicated in defense against parasites, we could expect that females with low CMI response had also higher blood parasite loads. This is very important because hemogregarines are known to have adverse effects in this lizard species (Amo et al., 2004). However, we did not find significant differences between treatments in intensity of hemogregarines' infection between the initial and the final measure. This could be due to experi-

mental females tended to be initially more parasitized than control females. However, it remains possible that because many physiological processes in lizards depend on optimal body temperature (Huey, '82; Stevenson et al., '85), and if low temperatures also implied a decrease in CMI response, there might be a subsequent increase of deleterious effects of parasites on body condition, even if parasite load did not change. Thus, we cannot conclude whether low temperatures per se or due to the effects of parasites caused a direct effect on body mass. Our data did not allow us to test this hypothesis, and hence, further studies are needed to reveal more physiological costs of refuge use that had not been considered until now.

The poor body mass of females that spent longer times at low temperatures may have significant effects on their fitness. Previous results showed that females of other lizard species in poor body condition produced offspring of small size (Shine and Downes, '99; but see also Gregory and Skebo, '98), and body size of neonate lizards can affect their probability of survival (e.g., Ferguson and Fox, '84; Sinervo et al., '92). Furthermore, females parasitized by blood parasites also showed reduced fat stores and produced smaller clutches (Schall, '83). Therefore, females forced to perform anti-predatory behaviors such as refuge use very frequently because they inhabit high risky areas (i.e., with a high predator density or with a high pressure by human tourists, at which lizards respond as to predators, see Amo et al., 2006), may incur physiological costs and negative consequences for the offspring. This deleterious effect of increased predation risk on body condition of females may endanger *I. cyreni* populations inhabiting risky areas.

Predation risk decreases as time spent in the refuge increases because the probabilities that the predator has left the area, or that it has focused its attention in another prey, increase in the course of time. In contrast, costs of refuge use increase when increasing time spent in the refuge (Martín and López, '99b). However, when prey is submitted to a second successive attack, risk should be considered higher than in the first attack due to predator persistence. Furthermore, after a second attack costs of refuge use should be greater than after the first attack because prey have previously spent some time inside the refuge (Polo et al., 2005). In this context, our results suggest that females are able to modify refuge use according to their body condition, with females in worse body mass decreasing time spent hidden

inside cold refuges, whereas females with a good body mass remained hidden for longer. This result remained constant in both attacks. Therefore, even though predation risk, but also the costs, were lower in the first attack, experimental females with worse body mass left the refuge sooner than control females. In this way females may be able to cope with predation risk while decreasing physiological costs of refuge use. These results agree with theoretical models (Sih, '92, '97; Martín and López, '99b) and previous studies with barnacles (Dill and Gillett, '91), polychaete worms (Dill and Fraser, '97), fishes (Krause et al., '98, but see also Dowling and Godin, 2002) or birds (Koivula et al., '95). Furthermore, the dependence of embryos of constant temperatures (Muth, '80) may force females to maintain optimal body temperatures in order to maximize the developmental rates of offspring (Mathies and Andrews, '97; Shine, 2004). In this way, females may shorten the incubation period and therefore decrease costs of reproduction (Shine, '80, '83; Seigel and Fitch, '84; Shine and Downes, '99). Shorter incubation periods may increase offspring fitness in areas with limited environmental temperatures because juveniles may have more time for growing before hibernation (Mathies and Andrews, '97; but see also Shine and Olsson, 2003). An alternative hypothesis might be that thermal inertia for females with worse body mass was lower than for females with greater body mass. Thus, females with worse body mass could get cold quicker than females in better body condition. Nevertheless, in any case, females would be adjusting refuge use to body mass.

To summarize, refuge use is costly for pregnant female lizards in terms of body condition due to time spent at low temperatures. However, our results suggest that lizards were able to modify their refuge use in relation to their body condition, with lizards with worse body mass having shorter hidden times.

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